FEBS 18225 FEBS Letters 404 (1997) 37–40

The transmembrane protein tyrosine phosphatase α dephosphorylates the insulin receptor in intact cells

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Received 16 January 1997

Abstract Protein tyrosine phosphatases (PTPs) are key regulators in a variety of signal transduction processes. However, substrates for most PTPs have not been determined. In a previous report, we demonstrated that in a transient expression system the intracellular phosphatases PTPs 1B and TC preferentially dephosphorylated the precursor form of several receptor tyrosine kinases. In this paper we show that the dephosphorylation of kinase precursors is a specific feature of PTPs 1B and TC that is not shared by two other intracellular PTPs, PTPH1 or SHP-1. By contrast, the receptor phosphatase PTPα preferentially dephosphorylated the β-subunit of the insulin receptor localized on the cell surface. The insulin receptor was a better substrate for PTPα than for other receptor type PTPs. We conclude that the intracellular PTPs 1B and TC regulate the autophosphorylation of receptor tyrosine kinases during their posttranslational processing while receptor type PTPs regulate the mature, cell surface localized receptor tyrosine kinases.

Key words: PTPα; Intracellular PTP; Insulin receptor; Dephosphorylation

1. Introduction

Identification of protein tyrosine phosphatase (PTP) substrates is of major importance to define the physiological role of PTPs. In vitro, several substrates of PTPs have been used to study target recognition and regulation of the catalytic activity, however, the results may depend on the specific substrates and assay conditions [1]. On the other hand, overexpression of PTPs in cell lines can be toxic [2] or may lead to a shift in the expression pattern of seemingly unrelated TKs or PTPs [3]. Nevertheless, together with in vitro binding studies this approach has allowed the identification of SH2 domain binding partners and substrates for the tyrosine phosphatases SHP-1 and SHP-2 [4–6]. The results obtained could be confirmed with mouse strains carrying SHP-1 mutant genes [7]. Overexpression of the receptor type PTPα in embryonic carcinoma cells activates the cytoplasmic tyrosine kinase src by dephosphorylating the inhibiting carboxy-terminal tyrosine [8]. Similarly, dephosphorylation of the carboxy-terminal tyrosine by PTP CD45 activates the Lck kinase and thereby the T-cell receptor complex. Only extracellular domain binding partners have been identified for other receptor type PTPs. PTPμ interacts in a homophilic manner with the PTPμ extracellular domain present on neighbouring cells, and the extracellular part of PTPβ binds to the GPI anchored protein contactin on neuronal cells [9–11]. Intracellular substrates for these phosphatases have not been identified.

For the intracellular PTPs 1B and TC which have been found to associate by their carboxy-terminal amino acids with the endoplasmic reticulum (ER) no specific substrates have been found [12,13]. We have previously shown that localization to the ER confines the activity of PTPs 1B and TC towards phosphotyrosine residues on receptor tyrosine kinases (RTKs) that share this intracellular compartment while being processed whereas the mature receptors are no targets for both PTPs [14]. This observation implies that PTPs 1B and TC are capable of dephosphorylating phosphotyrosine residues within different amino acid sequences which is supported by in vitro assays and crystallization studies that show an easily accessible catalytic center of PTP1B [15]. We have furthermore investigated the interaction of receptor tyrosine kinases with CD45. The capability of CD45 to dephosphorylate RTKs was mainly confined to c-Kit and no discrimination was seen between the intracellular and the cell surface localized receptor. In another study, we established a system that selected PTPs which negatively regulate the insulin receptor (IR) tyrosine kinase [16]. BHK cells overexpressing the IR did not grow and detached from the substratum when treated with insulin. Since overexpression of PTPα and to a lesser extent PTPs ε and TC overcame this inhibiting effect we decided to investigate the interaction of PTPα with the IR in more detail. In the present study, we compared the capability of the transmembrane PTPs α and ε and the intracellular PTPs 1B and TC to dephosphorylate the IR upon transient expression in 293 cells or in BHK cell lines. We found that in contrast to these ER associated PTPs the transmembrane PTPs are highly specific for the cell surface localized form of the IR.

2. Materials and methods

2.1. DNAs, antibodies and cell lines

All cDNAs were cloned into a cytomegalovirus early promoter based expression plasmid [17] and have been described earlier [14,16]. Polyclonal antisera were generated in rabbits using KLH coupled carboxy-terminal peptides of the IR, IGF-1R, PTPα and PTPε and an amino-terminal peptide for PTPs 1B and TC. Monoclonal antibody 83-14, directed against the extracellular domain of the IR, has been described [18]. The generation of BHK cell lines overexpressing the IR or the IR and PTPs α or TC has been described in [19].

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PII S0014-5793(97)00080-X

2.2. Transient expression experiments

Experiments were performed as described [14]. Briefly, cells were grown in F12/DMEM, 50:50, with 10% fetal calf serum, 2 mM L-glutamine, and antibiotics. 2 μg of plasmid DNA for receptor or phosphatase were transfected into 3×10^5 cells/10-cm^2 well according to the protocol of Chen and Okayama [20]. 18 h after the addition of DNA precipitate, cells were washed once and supplied with fresh medium containing 0.5% serum. 24 h later, cells were stimulated with ligand (100 ng/ml; insulin or IGF-1) for 10 min and then lysed in 200 μl lysis buffer (50 mM HEPES, pH 7.2, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 100 mM NaF, 10 mM sodium pyrophosphate and 1 mM sodium orthovanadate). The lysate was centrifuged for 2 min at $12\,500\times g$, 30 μl of the supernatant taken, sample buffer added, proteins boiled for 10 min and analyzed by SDS-PAGE and immunoblotting. The cell surface localized fraction of the IR was determined by incubation of the cells at 4°C for 30 min and addition of 10 μg/ml of antibody 83-14 and 100 nM insulin. After incubation for an additional 1 h, the cells were washed 5 times with cold medium and lysed as described.

3. Results

In an earlier study we had suggested that the intracellular PTPs 1B and TC preferentially dephosphorylate the precursor form of RTKs [14]. We hypothesized that transmembrane PTPs could exert their effects on the processed receptors localized at the cell surface and therefore compared the capacity of PTPs α and ε and PTPs 1B and TC to dephosphorylate the IR and the insulin-like growth factor 1 receptor (IGF-1R) in 293 cells transiently overexpressing these proteins. Equal amounts of IR expression plasmid were transfected either together with empty vector or with the respective PTP expression plasmid into 293 cells. The cells were made quiescent by incubation with medium containing 0.5% fetal calf serum, stimulated with insulin for 10 min and lysed in a Triton X-100 containing lysis buffer. Aliquots of the lysates were combined with Laemmli buffer, boiled for 5 min and proteins were separated by SDS-PAGE, transferred to nitrocellulose and incubated with phosphotyrosine specific antibody. After detection of phosphorylated proteins, the antibody was stripped off the nitrocellulose and overexpression of proteins was verified by reblotting with protein specific antibodies (Fig. 1). IR and IGF-1R assemble as $\alpha_2\beta_2$ heterotetramers and, when overexpressed, the phosphorylated β-subunit (95 kDa) is detected whereas the unphosphorylated extracellular α-subunit cannot be visualized. Autophosphorylated precursor forms with different degrees of glycosylation are detected at 170–180 kDa because the high expression level (up to 2×10^6 receptors per cell) overloaded the receptor processing system.

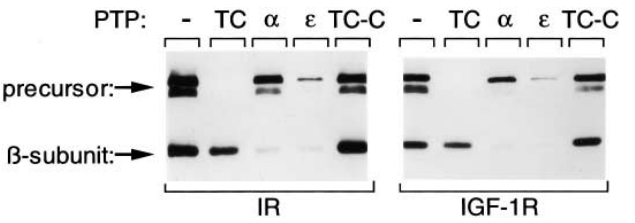

Fig. 1. Coexpression of the IR or IGF-1R with intracellular or transmembrane PTPs. Human embryonic kidney 293 cells transiently overexpressing the indicated proteins were stimulated with insulin for 10 min and lysed. Equal amounts of lysate were size separated by electrophoresis, proteins transferred to nitrocellulose and blotted with phosphotyrosine antibody. IR, insulin receptor, IGF-1R, insulin like growth factor receptor-1, TC-C, catalytically inactive PTPTC.

Cooverexpression of PTPTC abolished tyrosine phosphorylation of receptor precursor whereas the β-subunit was phosphorylated almost as strongly as in control cells. By contrast, coexpression of the IR/IGF-1R with PTPs α or ε reduced tyrosine phosphorylation of the β-subunit to background levels whereas phosphorylation of the precursors was only partially reduced but occurred to a greater extent in PTPε overexpressing cells. Coexpression of catalytically inactive PTPTC had no effect on receptor tyrosine phosphorylation.

Since dephosphorylation could also have been occurred while PTPα and the IR were processed and on their way to the cell surface, the degree of IR dephosphorylation at the cell surface was investigated more closely. Cells were transfected as in the previous experiment but before stimulation cooled to 4°C to block receptor internalisation. Insulin was added as indicated, and all samples were incubated with monoclonal antibody 83-14 directed against the extracellular domain of the IR. After 1 h the cells were washed several times to remove unbound antibody, lysed, and the antibody-IR complex was collected with protein A Sepharose. Antibody 83-14 and protein A Sepharose were added to the supernatant to immunoprecipitate the residual IR. The phosphotyrosine content of the IR was detected by blotting with phosphotyrosine antibodies. As shown in Fig. 2A, the β-subunit but not the precursor form is localized at the cell surface. Whereas coexpression of PTPα did reduce the phosphorylation of the β-subunit coexpression with PTP1B had no effect. Fig. 2B shows the phosphotyrosine content of the residual IR that is not localized at the cell surface. The IR precursor is immunoprecipitated with low efficiency but clearly dephosphorylation occurred only when PTP1B was coexpressed. Phosphorylation

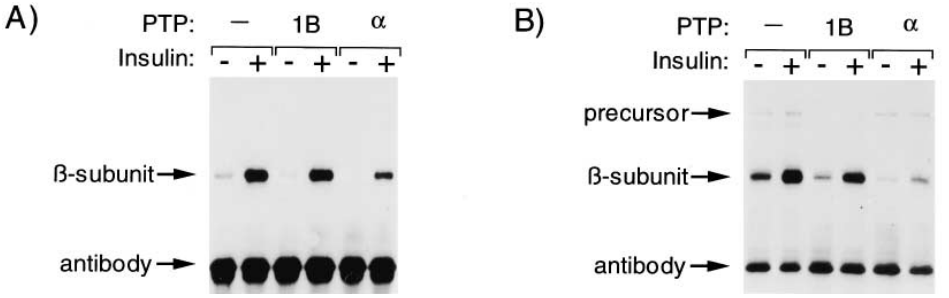

Fig. 2. Interaction of the IR and PTPs in different cellular compartments. 293 cells transiently overexpressing the IR alone or together with PTP1B or PTPα were cooled to 4°C, incubated with monoclonal antibody 83-14 and insulin as indicated for 1 h, washed, lysed and the antibody-IR complex collected (A). IR remaining in the supernatant was immunoprecipitated with antibody 83-14 (B). An immunoblot with antibody directed against phosphotyrosine is shown.

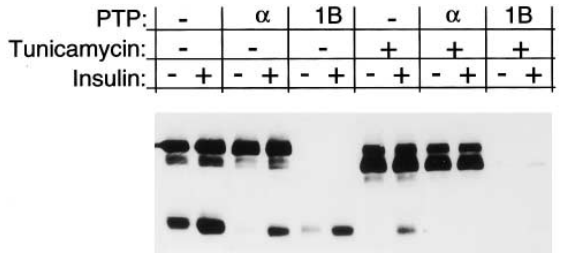

Fig. 3. Effect of inhibition of glycosylation on IR dephosphorylation. 293 cells transiently overexpressing the IR alone or together with PTP1B or PTPα were treated overnight with tunicamycin, stimulated with insulin for 10 min as indicated and lysed. The phosphotyrosine content of the IR was analysed.

of the β-subunit was insulin dependent but also found in unstimulated cells because of the high number of expressed receptors which leads to spontaneous kinase activation. This spontaneous activation was reduced when PTPs were coexpressed, as was the phosphorylation of the β-subunit in insulin stimulated cells.

In a complementary approach we tested the effect of PTP cooverexpression on IR tyrosine phosphorylation in the presence of tunicamycin, an inhibitor of *N*-glycosylation (Fig. 3). Treatment with 10 μg/ml tunicamycin overnight increased the amount of phosphorylated 170 kDa precursor whereas the amount of processed β-subunit decreased. PTPα could not dephosphorylate the IR precursor but PTP1B dephosphorylated even the unglycosylated precursor form.

A similar experiment was performed using BHK cells stably overexpressing 5×10^6 IR or the IR and in addition PTPα or PTPTC. As shown in the left part of Fig. 4A, in cells not treated with tunicamycin the main tyrosine phosphorylated protein was the IR β-subunit. Phosphorylation was clearly ligand dependent and reduced when PTPα or PTPTC were coexpressed. Since the amount of IR in these cell lines varies slightly the reduction of β-subunit phosphorylation does not reflect the activity of the coexpressed phosphatase (Fig. 4B). The 170 kDa phosphoprotein in cells not treated with tunicamycin probably represents IRS-1 since it was not detected by reblotting with IR specific antibodies. After treatment with tunicamyin a strongly phosphorylated protein appeared independent of insulin stimulation that migrated at the same position as the IR precursor. Because *N*-glycosylation was inhibited this precursor migrated faster than in untreated cells (Fig. 4B). As predicted from the transient expression experiments, however, the precursor was not phosphorylated in the presence of PTPTC.

To evaluate the specificity of PTPα or PTP1B activity in the transient expression system we coexpressed the insulin receptor substrate-1 (IRS-1) and other PTPs together with the IR as shown in Fig. 5. PTPs CD45 and LAR are representatives of the family of transmembrane PTPs while PTPH1 and SHP-1 are found intracellularly. None of these PTPs was able to dephosphorylate significantly the IRS-1, the IR precursor or the β-subunit although the total phosphotyrosine content of proteins was reduced, probably because some unspecific localization of PTPs occurred. These results confirm our data obtained in BHK-IR cells [19] and support the hypothesis that transmembrane PTPs are regulating RTKs localized at the cell surface while PTPs 1B and TC are responsible for controlling the tyrosine phosphorylation state of RTK precursors.

4. Discussion

In the present report, we have investigated in more detail findings from previous studies that suggested a general role for ER associated PTPs to keep RTKs quiescent during their posttranslational processing. Since PTPs α and ε were found to act as negative regulators of the IR tyrosine kinase we compared the dephosphorylating activity of these PTPs towards the IR with that of PTPs 1B and TC. In total cellular lysates from 293 cells transiently overexpressing these proteins dephosphorylation of the coexpressed IR by PTPα occurred preferentially at the processed β-subunit (Figs. 2 and 3). At the cell surface, only PTPα but not PTP1B coexpression reduced phosphotyrosine content of the β-subunit. When coexpressing IR and PTPα, dephosphorylation of the β-subunit was more efficient in total cell lysate than at the cell surface, indicating that PTPα and the processed IR both segregate

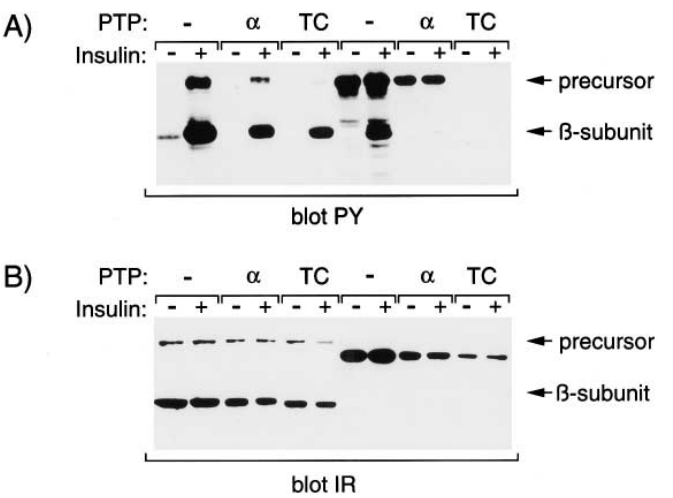

Fig. 4. Inhibition of glycosylation in IR overexpressing BHK cells. Confluent cells were made quiescent, treated with tunicamycin (right part) or not (left part), stimulated with insulin as indicated and lysed in Triton X-100 buffer. The phosphotyrosine content of the IR was detected by blotting with phosphotyrosine specific antibodies (A). Antibodies were then stripped off the filter and the IR detected by specific antibody (B).

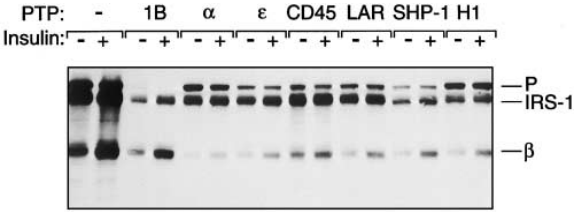

Fig. 5. Dephosphorylation of the IR and IRS-1 by an extended set of PTPs. 293 cells transiently overexpressing the IR and IRS-1 alone or together with different PTPs were stimulated with insulin for 10 min as indicated. Analysis of phosphotyrosine content of the proteins from total cell lysate is shown.

from compartments shared with PTP1B before reaching the cell surface. Tunicamycin treatment should retain PTPα in the compartment where early glycosylation takes place. In a similar compartment the IR precursor should localize. Nevertheless, PTPα can act on the IR precursor neither in transiently expressing 293 cells nor in BHK cell lines, suggesting either a different microenvironment for these two proteins or the requirement for PTPα to be activated by some posttranslational event, for example, the interaction with a chaperone. In vitro, however, the PTPα precursor is active [21], and in our experiments (data not shown). The preference of PTPs 1B and TC to dephosphorylate the IR precursor form is not a general feature of intracellular PTPs. PTPH1 contains an ezrin domain and localizes probably to cytoskeleton structures while SHP-1 contains an SH2 domain and binds to a specific subset of phosphorylated tyrosines. Consequently, they are not positioned to dephosphorylate the IR precursor. On the other hand, the transmembrane PTPs CD45 and LAR do not act preferentially on the IR β-subunit nor do they show a higher capacity to dephosphorylate the IR than PTPα. Both PTPs were not able to rescue BHK-IR cells treated with insulin although reduced expression of PTPLAR in rat hepatoma cells has been reported to cause enhanced tyrosine phosphorylation of at least three RTKs including the IR [22]. Therefore, hepatoma specific activation factors may be required to activate PTPLAR or PTPLAR does not interact directly with the IR. Taken together, these data support the identification of PTPα as a negative regulator of the IR kinase and suggest the cell surface as the place of interaction.

Acknowledgements: We are grateful to S. Müller for expert technical assistance and I. Sures for critical reading of the manuscript.

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